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Anatomy and Histochemistry of Araticum (*Annona crassiflora* Mart.) on Three Annonaceae Rootstocks

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Abstract

The cultivation knowledge about the Cerrado native fruits is incipient. These plants are found in wild conditions and its fruits are obtained through extraction, as the case of araticum (*Annona crassiflora* Mart.), which is a species with the great economic potential. This plant propagation by grafting, among other methods, has proved problematic. Possible incompatibility causes were investigated using histochemical and anatomical studies. Transverse and longitudinal stems sections were analyzed in the araticum grafting area on rootstocks of araticum-de-terra-fria (*Annona emarginata* (Schltdl.) H. Rainer "Terra-fria"), biribá (*Annona mucosa* (Jacq.) Baill), and soursop (*Annona muricata* L.). Araticum graft survival rate was low with these rootstocks, which seems to be associated with anatomical and histochemical factors. The periderm and pith are more developed in araticum than in the other plants, which affects the alignment and juxtaposition of the cambium and vascular bundles in the graft area, hindering a successful graft. The histochemical reactions for phenolic compounds detection were very strong in cortical parenchyma, pith and xylem fibers of araticum (*A. crassiflora*). The presence of phenolic compounds is increased depending on the cut for grafting and the formation of these compounds is evidenced as an important limiting factor for successful grafting. It is recommended practices for reducing these compounds and a larger diameter of the graft, as a mean to overcome these anatomical difficulties.

Keywords

Phenolic Compounds, Incompatibility, *Annona* sp.

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1. Introduction

The Cerrado region covers 204 million hectares, mainly distributed in the states of Minas Gerais, Goiás, Mato Grosso, Mato Grosso do Sul, Tocantins, Bahia, Piaui, Maranhao, and the Federal District, corresponding to approximately 22% of Brazil's territory [1]. The incorporation of this region to the agricultural production area has enabled the expansion and diversification of Brazilian agriculture. However, the lack of knowledge of the potential use of natural resources and environmental protection laws disrespect, burning the savanna and intensity of farming has caused irreparable damage, to the soil, fauna, flora and water resources, compromising the ecosystem sustainability and by placing many animal and plant species in risk of extinction, especially the native fruits [1].

According to [2], among the native Cerrado fruits that have not been domesticated, the araticum (*Annona crassiflora* Mart.) is a species with the great economic potential, which can be used in association with other species and also in preservation areas, generating income to producers and ensuring minimal impact to the environment.

Araticum has a low germination rate of seeds and, according to [3], they present a hard and thick endosperm, but the hilum has dense conspicuous caruncle and a hole that allow air and water exchange. For such a limited sexual propagation species an asexual propagation method is recommended [4]. The intergeneric grafting of the araticum would be an option for this Cerrado species domestication, as low seed germination and slow development in nursery conditions are factors that hinder this species propagation [2] [5].

The grafting compatibility is understood as one in which the union is successful and that has the satisfactory development forming a single plant composition. When that does not happen, we have a graft incompatibility [6].

Affinity includes the plant's morphological and physiological aspects. The morphological and anatomical affinities as well as tissue formation affinities refer to the two plants' conductive vessels which are compatible for showing similar diameters and in aproximately equal number. The physiological affinity is related to the quantity and composition of the sap [7]. According to [8], toxins that inhibit the formation of callus tissue can occur. Peroxidase is an enzyme whose specificity is to act on peroxides, mainly hydrogen, breaking them down and releasing oxygen [9].

This study aims to evaluate the graft incompatibility in propagation of araticum (*A. crassiflora* Mart.) on three rootstocks: araticum-de-terra-fria (*Annona emarginata* (Schltdl.) H. Rainer "Terra-fria"), biribá (*Annona mucosa* (Jacq.) Baill) and soursop (*Annona muricata* L.), all belonging to Annonaceae.

2. Material and Methods

2.1. Description of Study Sites

The graft's anatomical and histochemical analysis were performed in the Laboratory of Plant Anatomy, in the Department of Botany, Faculty of Biology, University of Brasília.

2.2. Cautions during and after Grafting

The rootstocks remained in a nursery with 50% shade screen and sprinkler irrigation once a day, suspending it on rainy days. The cuts were made with carbon steel blades sharpened and cleaned with soap and water. The cuts were made quickly and accurately, uniting the graft and rootstock with plastic tape. The araticum's scion collected had a diameter similar to the rootstock, for better juxtaposition of the cambium tissue, and were cut into sections containing about 4 buds and wrapped in paraffin film for subsequent use in grafting. The grafted plants were covered with transparent plastic bags, measuring 30 cm long by 5 cm wide. Plants were always inspected for weed control and pests and diseases monitoring. Spraying with insecticides, acaricides or fungicides were not necessary during the experiment conduction and plants were not fertilized after grafting.

2.3. Anatomical and Histochemical Evaluation

Slides with sections were obtained by microtome table. The grafting union area was underwent to anatomical histochemical analysis. Anatomical characterization was carried out using transverse and longitudinal sections of stem in area of grafting, performed in microtome table and clarified for 30 minutes in a solution of 50%. sodium hypochlorite. The sections were dyed for 1 min with 1% safranin and alcian blue in 5:1 ratio with water. The sections were placed on glass slide and coverslip with water.

For histochemical tests, samples of stem in the graft area were fixed in formaldehyde, acetic acid and ethyl-

alcohol (FAA) 50% and stored in a 50% alcohol solution for later use in testing [10]. This material was sectioned freehand on a table microtome and subjected to Iron (III) chloride and potassium dichromate to check the location of phenolic compounds, followed by an optical microscope observation. Potassium dichromate detects the presence of general phenolic compounds, keeping the cuts in an aqueous solution of potassium dichromate to 10% for 30 - 60 minutes [11], then washing in water and continuing to put in water for optical microscope observation. The color that indicates the presence of phenolic compounds is reddish brown. Iron (III) chloride operates in other classes of simple phenolic compounds [10]. Drops of reagent at 10% solution were put in the cuts, keeping them for 15 minutes and then rinsing them in water. The blue-black or dark green color shows the phenolic compounds.

For these parameters analysis, boards were made with pictures taken from slides observed under a optic microscope.

3. Results and Discussion

3.1. Scion and Rootstock Histochemical Analysis

In araticum, the presence of phenolic compounds is increased by cutting and these compounds formation is the main limiting factor of grafting success. Phenolic compounds are associated with plant defense against pathogens and herbivores [12]. The scion araticum showed positive reactions to Iron (III) chloride and potassium dichromate, wich react with phenolic compounds. Reactions to the Iron (III) chloride were found in xylem fibers in and cortical parenchyma. For potassium dichromate, reactions were found in pith, xylem vessel elements and phloem fibers in cortical parenchyma (**Table 1**). The soursop rootstock showed the reaction of Iron (III) chloride in periderm, cortical parenchyma, fibers, dilated parenchyma rays of the phloem. For potassium dichromate, it showed weak responses in the cortical parenchyma (**Table 1**). The Iron (III) chloride reaction on araticum-de-terra-fria rootstock showed positive response in periderm and cortical parenchyma. With potassium dichromate it showed weak reaction in the cortical parenchyma in the sieve tube elements of phloem and in the cambium

Table 1. Ocurrence of phenolic componds in the stem tissues of *Annona crassiflora*, *Annona muricata*, *Annona mucosa*, and *Annona emarginata* var. terra fria evaluated by the potassium dichromate test and Iron (III) chloride. (+) presence; (−) absence; (+/−) weak reaction Legend: Iron (III) chloride = ICIII; PD = potassium dichromate.

Tissues/structures		*Annona crassiflora*		*Annona muricata*		*Annona mucosa*		*Annona emarginata* var. terra fria	
		Phenolic compounds							
		ICIII	PD	ICIII	PD	ICIII	PD	ICIII	PD
	Periderm	+	+	+	+	+/−	+/−	+	+
	Cortical parenchyma	+	+/−	+	+	−	−	−	−
Phloem	Fibers	−	−	−	−	−	−	−	−
	Sieve tube element	−	−	−	−	−	−	−	−
	Radial parenchyma	−	−	+/−	−	−	−	−	−
	Phloem ray	−	−	+	+	−	−	−	−
	Cambium	−	−	−	−	−	−	−	−
	Axial parenchyma	−	−	−	−	−	−	−	−
	Radial parenchyma	−	−	+/−	−	−	−	−	−
Xylem	Vessel element	−	−	−	−	−	−	−	−
	Fibers	−	−	−	−	−	−	−	−
	Inner phloem	−	−	−	−	−	−	−	−
Pith, parenchyma, fibers		+	+	−	−	−	−	−	−

zone. The biriba rootstock showed weak responses on the phloem sieve tube with Iron (III) chloride. In biribá rootstock, there were positive reactions to the Iron (III) chloride, showing formation of phenolic compounds in the periderm and phloem fibers.

3.2. Histochemical and Anatomical Analysis of Araticum Grafting on Three Rootstocks

Due to the low araticum grafting survival rate on rootstocks, we proceeded the anatomical and histochemical study in the grafting area, to assist in addressing the causes of these low rates. Histochemical tests revealed the presence of phenolic compounds in parenchyma cells of the grafting area, being very pronounced in araticum tissues. During the development of the experiment, the rootstocks sprouted in the area below the graft because apical dominance breaking by grafting methods. This fact shows that the rootstocks were seen to be stronger and able to develop. Most grafts that did not work, presented bud necrosis, dry-looking, with little time after grafting. Two grafts showed buds swelling at 45 days after grafting, but a graft, with the English method showed a little development of the bud and then immediately died. The other graft, with the method simple English, was able to develop, but with little vigor.

According to [8], it is clear that anatomical similarities between scion and rootstock, the presence of cambium or direct contact is not sufficient to form a perfect union. The successful parts union in a graft is the result of the deposition and polymerization of cell wall material due to injury caused by cutting. Thus, it is classified as a passive event, which can occur in both compatible and in incompatible species, but the differentiation of vascular tissues in callus only occurs in compatible species. The phenolic compounds presence causes lack of response of the callus to growth promoters, so there is no differentiation and consequently no transport of water and nutrients from the rootstock to graft and *vice versa*.

It was noted differentiated araticum anatomy in relation to rootstocks, because it shows larger periderm and pith with the same diameter shoots, affecting the juxtaposition of cambium and vascular bundles and making the successful grafting difficult (**Figures 1(A)-(D)**). It was observed that the grafting area had no evidence of callus formation, except in a case of araticum grafting on the araticum-de-terra-fria rootstock and according to [13], there is evidence of incompatibility (**Figure 1(E)**).

The presence of tyloses was also described by [14] in grafting of camu-camu (*Myrciaria dubia*) on rootstock of guava and surinam cherry, which is characterized by the vessel elements obstruction, indicating incompatibility type located and immediate. According to [8], this type of incompatibility toxins act as inhibitors of promoting compounds of graft union, such as auxins. For [9], the processes involved in cell walls lignification are mainly responsible for a solid union, while compounds that inhibit the lignin formation and the middle lamella cells zone contact of the graft appeared to be co-responsible for the incompatibility symptoms.

Since phenolic compounds are essential to the lignin synthesis, they represent an important cell walls component whose structure varies depending on constitution of phenolic compound type, between plant species and between cell types [9]. Most studies on toxins acting as factors of graft incompatibility is based on the presence of specific compounds such as cyanogenic glycosides (prunasin), polyphenols and peroxidase [8]. The peroxidase is related to the lignin formation and can affect the union process, weakening the graft by the absence of lignification or hindering the translocation of water (by increasing lignification), leading to incompatibility [8] [9].

In the araticum grafting on araticum-de-terra-fria rootstock it was noticeable that the union was not complete and only a part of the grafting area formed a differentiated callus. Even 12 months after the procedure, the grafted plant showed little vigour and swelling in the area above grafting area, typical symptoms of morphological incompatibility. According to [15], both parts must present cells, shape and consistency similar, since there is no cells exchange, in other words, each tissue continues to produce its own cells (**Figure 1(E)**).

It can be observed in **Figure 1(E)** that the cambium area is misaligned due to the anatomical difference shown between the rootstock and the scion. Araticum has periderm and pith wider than araticum-de-terra-fria, causing an alignment failure of cambium tissue when performing grafting. As the cambium area is thin, consisting of few layers of cells, can often become misaligned, impairing the establishment of a histological continuity between the rootstock and the graft, without the formation of new tissue between them. In stem grafting bud of "Valencia" orange on rootstock of "Cravo" lemon, [16] observed that there is no need of juxtaposition of the vascular tissue, with proliferation of parenchyma cells and sorting for the continuity of vascular tissues. [17] observed that, when there is an overlap of the vascular tissue, the reconstitution of the vascular system appeared

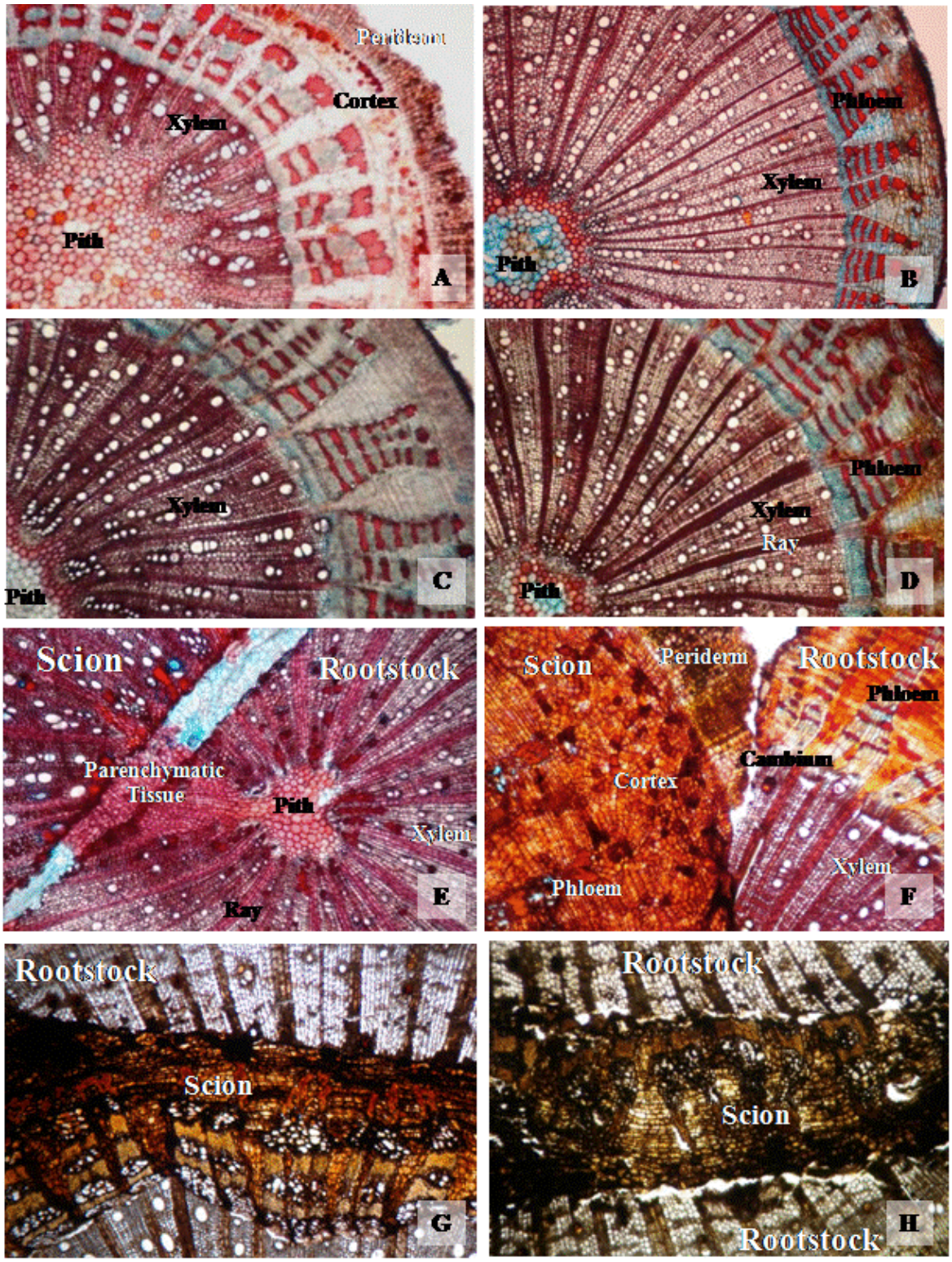

Figure 1. Transverse section of the stem. (A)-(D) Anatomical differences between species. (A) *Annona crassiflora* Mart.; (B) *Annona emarginata* (Schltdl.) H. Raine; (C) *Annona mucosa* (Jacq.) Baill.; (D) *Annona muricata* L.; (E) Stem in secondary growth of *A. crassiflora* grafting region on *A. emarginata*; (F) Absence of callus on the grafting of *A. crassiflora* on (A) muricata region; (G) (H) Phenolic compounds in *A. crassiflora* on *A. mucosa* (orange colour: potassium dichromate, black: Iron (III) chloride.

to be more efficient and rapid on grafted coffee. The long period of domestication and grafting in orange is not comparable with the little knowledge we have about araticum, where there seems to be a requirement to align the cambium, such as with coffee.

The cambium is a meristematic area, therefore able to divide, which is important for the new tissues formation such as xylem and phloem, which are responsible for crude and elaborated saps transportation, respectively [12] [18]. Without the union of these tissues by grafting, decreases the probability of fixation. The union can also be favored by the dedifferentiation of parenchyma cells near the exchange. These dedifferentiated cells can form a new continuous cambium between rootstock and graft. It is observed in **Figure 1(E)** that the callus formed showed parenchymatic characteristics without differentiation of new vessel elements and sieve tube even with 12 months after grafting. The callus provided a good link between rootstock and graft, but did not allow the continuity of the vessel elements, which is essential for the graft survival.

In the araticum grafting on the soursop rotstock, it was observed that the grafting region showed no hint of callus formation, suggesting that there was incompatibility localized and immediate. Araticum presents different anatomy in relation to soursop, with more developed pith and periderm compared to soursop, making the juxtaposition of parenchymatous tissue and cambium difficult. There was a phloem reaction to Iron (III) chloride and in the araticum cut surface, besides the presence of tyloses in soursop, both identifiable by the reaction to ferric chloride and with potassium dichromate (**Figure 1(G)**).

In the araticum grafting on the biribá rootstock, the phenolic compounds formation were found near the grafting area, in the secondary phloem and secondary xylem of both species (**Figure 1(G)** and **Figure 1(H)**). [14] noted the absence of cell division formation in the graft area and obstruction of the vessel elements, called tyloses, in camucamu grafting (*Myrciaria dubia* (Kunth) McVaugh) on rootstocks guava and surinam cherry. According to [19] small amounts of phenols are capable of producing limited disorders at the interface of two cells. It was observed blackening of araticum tissue when grafted, indicating that there was oxidation of the tissues due to phenolic compounds. According to [20] phenolic compounds in the callus were present in greater quantity in incompatible combinations, as compared to compatible.

According to [21] 4 weeks after grafting *Swainsonia formosa*, continuous vascular tissues were observed from the rootstock to graft in successful grafts. As for incompatible combinations, parenchyma cells (callus) were observed between the vascular bundles of the rootstock and the graft, and there were no links in the region of vascular graft union. These data are consistent with the results obtained by [22], which had low survival rates of grafting soursop on seven rootstocks, considering that *Annona glabra*, *Annona squamosa*, *Annona emarginata* obtained very low survival rates, less than 5% and reaching 0% on *Annona* glabra.

An incompatibility in *Uapaca kirkiniana* Müell Arg. was studied by [23] and was attributed to the high concentration of phenols, flavonoids, anthocyanins and their derivatives at the interface of graft area.

4. Conclusion

Araticum grafting on rootstocks of soursop, biribá, araticum-de-terra-fria proved to be unfeasible due to incompatibility with the rootstocks tested. Phenolic compounds were identified in the region of grafting, especially in parenchymatic cells in araticum stems.

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Abbreviations

C: cambium
IC: iron chloride III
PC: parenchymatous callus
CX: cortex
PD: potassium dichromate
V: vessel
P: phloem
Fi: fiber
SP: secondary phloem
I: idioblasts
M: pith
P: periderm
PR: parenchymatic ray
LS: longitudinal section
CS: cross section
S + AB: safranin and alcian blue

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